PROTEASE IN AMMOCOETES ENDOSTYLE[1]

MARGARET CLEMENTS AND AUBREY GORBMAN

Departments of Zoology, Barnard College, Columbia University
New York 27, New York

It is now generally accepted that there is at least one proteinase associated with the breakdown of thyroglobulin in the thyroid gland. The action of such an enzyme is necessary to release thyroxine from protein, so that it may be able to diffuse through the follicular epithelium into the blood. The enzyme has been demonstrated in thyroids of rats and guinea pigs (Dziemian, 1943; De Robertis, 1941), man (De Robertis and Nowinski, 1946), and dog (Kamner *et al.*, 1950). From beef thyroid, Anson (1937) partially purified a proteolytic enzyme with properties of a catheptase; Weiss (1953) further characterized the beef protease biochemically. McQuillan and Trikojus (1953) were able to show the existence of a close thyroglobulin-enzyme association in hog thyroid and they devised a method for its purification.

Considerable interest attaches to the question of whether the larval thyroid gland of lampreys, the "endostyle," possesses a proteinase similar to that of thyroids of the higher vertebrates. The endostyle opens by means of a duct into the pharynx so that any thyroid-like secretion could be passed through it for digestion and absorption in more posterior parts of the gut. Thus, it would not appear to be essential for the endostyle to have an enzymatic thyroxine-freeing mechanism. On the other hand, when the lamprey larva undergoes metamorphosis, the duct to the pharynx is closed and the endostyle is in this way converted into a more or less typical follicular thyroid gland in the adult. Such closed follicles would require enzymatic hydrolysis of their contained thyroprotein. At what stage in these developments does the thyroidal proteinase appear? Does it precede the endostyle-thyroid transformation, or follow it? These are the questions that the present work has attempted to answer.

Materials and Methods

Because of the small size of the endostyle, it was necessary to employ a procedure especially adapted to the determination of the proteolytic enzyme on a micro level. The technic used for the measurement of enzyme activity was modified from the procedures of Anson (1937) and Kamner *et al.* (1950).[2] The animals used were the ammocoetes larvae of *Petromyzon marinus* collected in the Salmon River near Plattsburg, New York.

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Larvae, ranging in size from 13 to 16 cm. in length, were used. The lower half of the pharynx was removed from unanesthetized animals and the endostyle was dissected out and weighed. Two endostyles at a time were homogenized in a glass microhomogenizer containing 50 microliters (μl) of 60% aqueous glycerol. Additional glycerol solution was added until the total tissue concentration was 0.1 mgm./μl. This homogenate was refrigerated for 15 minutes at 4° C., and then distilled water was added to give a final tissue concentration of 0.05 mgm./μl. After careful stirring with a fine, glass rod, the mixture was again refrigerated for one-half hour. The homogenate was then centrifuged, and the supernatant, used for analysis, was drawn off as needed. Endostyles of four or five ammocoetes were used in each separate test.

A 2.5% aqueous solution of hemoglobin,[3] was made up not more than three days before use as the stock substrate material. The final solution of buffered substrate, prepared just before use, contained 4 ml. of 2.5% hemoglobin solution and 1 ml. of a solution of acid (and/or salt) of desired molarity. The pH was determined on the Beckman pH meter.

TABLE I

The effect of hydrogen ion concentration upon endostylar proteolytic activity. Incubation time: 5 hours

25 μl of homogenate (1.25 mgm. tissue) +150 μl of buffered hemoglobin, pH:	Micrograms of tyrosine freed per 1.25 mgm. tissue
2.00	0
2.60	0
3.42	25.5
3.90	35.4
4.00	36.2
4.30	27.4
5.00	14.0

A series of conical two-ml. Pyrex centrifuge tubes, each containing 10 μl (or more) of the homogenate supernatant, was prepared in groups of three, two experimentals and one control tube. Into each was pipetted 150 μl of the buffered substrate of the desired pH. To the controls, 275 μl of 0.3 *M* trichloroacetic acid (TCA) also was added. This proved to be as satisfactory as heat inactivation suggested by Kamner *et al.* (1950). These tubes were then stoppered and placed in a water bath at 37.0° $\pm$ 0.02° C. After the desired interval, the reaction in the experimental tubes was stopped by the addition of 275 μl of 0.3 *M* TCA and thorough mixing. Since filtering of these small volumes was not practical, the tubes were centrifuged at 3000 rpm. and the clear supernatant was analyzed for liberated tyrosine as follows: 200 μl of the supernatant of each tube were quantitatively transferred into a correspondingly marked cuvette. To this, 400 μl of 0.5 N NaOH was added in similar fashion and the cuvettes were inverted three times to insure complete mixing. One part of Folin-Ciocalteu phenol reagent[4] was

[3] Powdered Bovine Hemoglobin Enzyme Substrate Powder, Pentex Incorp., Kankakee, Illinois.

[4] Eimer and Amend, New York.

FIGURE 1. Proteolytic activity of endostylar enzyme as a function of time. Tissue concentration: 0.5 mgm./10 μl. of homogenate.

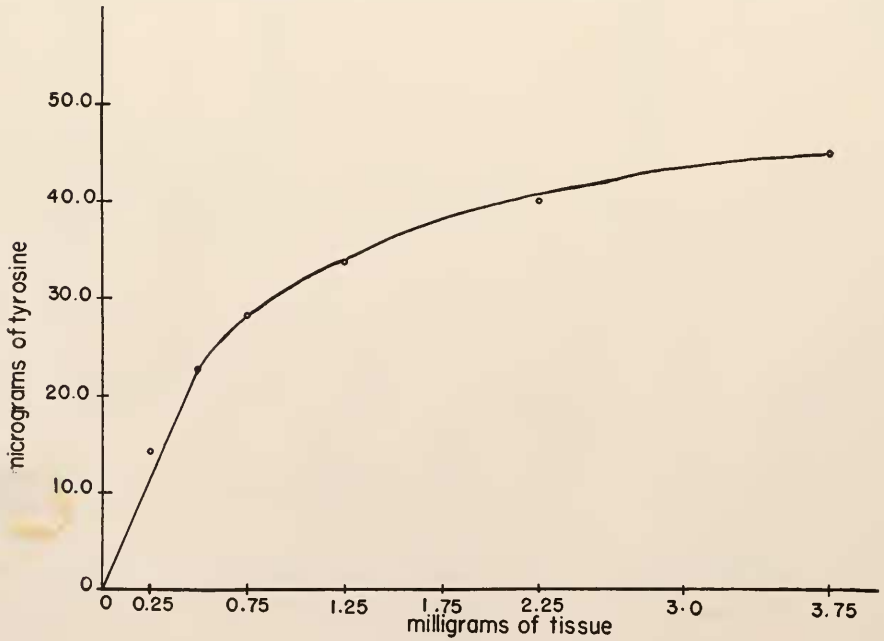

FIGURE 2. Proteolytic activity of the endostyle as a function of its concentration expressed as milligrams of tissue per unit volume. Incubation: 5 hours.

diluted with two parts of distilled water, and 150 μl of this dilution was added to each cuvette which was again inverted three times. The characteristic blue color produced in 15 minutes was measured in a Coleman, Jr. spectrophotometer at 700 λ and values were expressed as micrograms of tyrosine liberated by the homogenate after subtraction of the control values. The tyrosine standardizing solution was prepared from l-tyrosine, and the curve was made by reading spectrophotometric values corresponding to quantities ranging from 5 to 35 micrograms of tyrosine.

Results

The proteolytic activity of glycerol extracts of endostylar tissue first was measured as a function of the hydrogen ion concentration of the *in vitro* reacting system. Optimal activity for digestion of the hemoglobin substrate was found at pH 3.9–4.0, decreasing measurably on either side of this peak (Table I). It is interesting that this optimum is quite close to those found by others in studies of mammalian thyroidal protease.

To make possible further comparison of the ammocoetes endostylar protease with the thyroidal proteases of other animals, studies were made of the proteolytic activity of the enzyme as a function of time, and of its concentration. The data for both of these studies were confirmed by several repetitions, and two series of experiments are summarized in Figures 1 and 2. It may be seen that under the conditions employed, and within stated limits of time and enzyme concentration, proteolytic activity is a linear function of either of these two variables.

Discussion

Schneider (1879) first observed the histological changes during the transformation of the endostyle of metamorphosing larvae, and these observations led him to the conclusion that the endostyle is the forerunner of the well-defined thyroid gland of the adult. It was Dohrn (1875, 1886) and later, Leach (1939), who suggested that the endostyle might serve functionally in the production of a hormone or its precursor. They further believed that this secretion might pass through the pharynx to the alimentary canal where it could be absorbed. Recently, Sterba (1953) in an extensive study determined that the Type III secretory cells of the endostyle of *Petromyzon planeri* contributed to a large extent to the formation of the follicles of the adult thyroid. This supplied the basis for the correlation of morphology and function, for it was the Type III cells of *Entosphenus lamottenii* that accumulated radioiodine in radioautographic tests (Gorbman and Creaser, 1942). This ability of the endostyle to metabolize and store iodine demonstrates its thyroid-like function, even during the prolonged larval period. Further confirmation has been provided by Leloup and Berg (1954) who showed by means of radiochromatography that the endostyles of the larvae of *Petromyzon marinus* accumulate iodide, and form moniodotyrosine, and diiodotyrosine when maintained at 15° C. and kept at 2° C., they also form thyroxine. Evidently, the same cells that participate in follicular formation of the adult gland are able, long before, to elaborate the precursors of thyroxine as well as thyroxine itself.

Following Dohrn's suggestion (1886) that the endostyle is an organ of digestive secretion, Alcock, as early as 1899, tested the endostyle for ability to digest

fibrin. Although she found other parts of the digestive tract active in this respect, the endostyle did not, in her tests, digest fibrin. She concluded that the endostyle did not function as a source of digestive ferments. Since this negative report there has been no work produced to either confirm or contradict this conclusion. The data reported here seem to establish the presence of an active protease of the catheptase type in the endostyle of ammocoetes. Further, this enzyme is present in a concentration comparable to the protease in the thyroids of mice and adult lampreys (unpublished data).

The usefulness of the endostylar protease is difficult to assess in terms of thyroid function. Whether the endostylar secretion, particularly that of the iodine-metabolizing Type III cells, passes into the lumen by holocrine (Gorbman and Creaser, 1942) or merocrine (Sterba, 1953) means, it seems to move rapidly into the pharynx (Sterba, 1953). This would not seem to provide sufficient opportunity for enzymatic hydrolysis of the secreted iodoprotein. If not digested in the endostyle it appears more likely that digestion of the iodoproteins would be continued and completed in the intestine, and it would be followed by absorption of the hormone.

At any rate, it is reasonable to conclude that the thyroidal protease of lampreys is formed in the larval endostyle, and therefore it precedes follicular differentiation, when its presence would be obligatory for proper thyroidal function.

Summary

1. A proteolytic enzyme was demonstrated, by use of a new microanalytical technique, to be present in the endostyle of the ammocoetes larva of *Petromyzon marinus*.

2. The protease has an optimum pH of 4.0. The hydrolysis of hemoglobin (expressed as micrograms of tyrosine released) under the conditions employed, and within a period of three hours, is a straight line function of time. The hydrolytic activity of the endostylar enzyme is also a straight line function of enzymatic concentration, but within restricted and defined limits.

3. The probable site of action of the hydrolysis of the endostylar thyroprotein is discussed in regard to the usefulness of the investigated endostylar protease.

LITERATURE CITED

Alcock, R., 1899. On proteid digestion in ammocoetes. *J. Anat. Physiol.*, **33**: 612–637.

Anson, M. L., 1937. The estimation of cathepsin with hemoglobin and the partial purification of cathepsin. *J. Gen. Physiol.*, **20**: 565–574.

De Robertis, E., 1941. Proteolytic enzyme activity of colloid extracted from single follicles of the rat thyroid. *Anat. Rec.*, **80**: 219–230.

De Robertis, E., and W. W. Nowinski, 1946. The proteolytic activity of normal and pathological human thyroid tissue. *J. Clinical Endocrin.*, **6**: 235–246.

Dohrn, A., 1875. Der Ursprung der Chordaten und das Princip des Funktionswechsels. Leipzig.

Dohrn, A., 1886. Thyroidea bei Petromyzon, Amphioxus, und den Tunicaten. *Mitt. Zool. Sta. Neapel*, **6**: 49–92.

Dziemian, A. J., 1943. Proteolytic activity of the thyroid gland. *J. Cell. Compar. Physiol.*, **21**: 339–345.

Gorbman, A., and C. W. Creaser, 1942. Accumulation of radioactive iodine by the endostyle of larval lampreys and the problem of homology of the thyroid. *J. Exp. Zool.*, **89**: 391–401.

KAMNER, M. E., A. PERANIO AND M. BRUGER, 1950. Factors affecting the measurement of proteolytic activity of thyroid tissue. *Endocrinol.*, **46**: 353–358.

LEACH, W. JAMES, 1939. The endostyle and thyroid gland of the brook lamprey, *Ichthyomyzon fossor*. *J. Morph.*, **65**: 549–605.

LELOUP, JACQUES, AND OLGA BERG, 1954. Sur la présence d'acides aminés iodés (monoiodotyrosine, diiodotyrosine et thyroxine) dans l'endostyle de l'ammocoete. *C. R. Acad. Sci.*, **238**: 1069–1071.

MCQUILLAN, M. T., AND V. M. TRIKOJUS, 1953. Purification and properties of thyroid protease. *Australian J. Biol. Sciences*, **6**: 617–629.

SCHNEIDER, ANTON, 1879. Beiträge zur Anatomie und Entwicklungeschichte der Wirbelthiere. G. Reimer Verlag, Berlin. pp. 85–92.

STERBA, GUNTHER, 1953. Die Physiologie und Histogenese der Schilddrüse und des Thymus beim Bachneunauge (*Lampetra planeri* Bloch = *Petromyzon planeri* Bloch). *Wiss. Zeitschr.* Friedrich-Schiller-Universität, Jena. Math.-Naturwiss. Reihe, Heft 2, pp. 239–298.

WEISS, B., 1953. Peptidase and proteinase activity of beef thyroid tissue. *J. Biol. Chem.*, **205**: 193–203.